Epigallocatechin-3-Gallate Inhibition of Myeloperoxidase and Its Counter-Regulation by Dietary Iron and Lipocalin 2 in Murine Model of Gut Inflammation

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Green tea-derived polyphenol (−)-epigallocatechin-3-gallate (EGCG) has been extensively studied for its antioxidant and anti-inflammatory properties in models of inflammatory bowel disease, yet the underlying molecular mechanism is not completely understood. Herein, we demonstrate that EGCG can potently inhibit the proinflammatory enzyme myeloperoxidase in vitro in a dose-dependent manner over a range of physiologic temperatures and pH values. The ability of EGCG to mediate its inhibitory activity is counter-regulated by the presence of iron and lipocalin 2. Spectral analysis indicated that EGCG prevents the peroxidase-catalyzed reaction by reverting the reactive peroxidase heme (compound I:oxoiron) back to its native inactive ferric state, possibly via the exchange of electrons. Further, administration of EGCG to dextran sodium sulfate-induced colitic mice significantly reduced the colonic myeloperoxidase activity and alleviated proinflammatory mediators associated with gut inflammation. However, the efficacy of EGCG against gut inflammation is diminished when orally coadministered with iron. These findings indicate that the ability of EGCG to inhibit myeloperoxidase activity is one of the mechanisms by which it exerts mucoprotective effects and that counter-regulatory factors such as dietary iron and luminal lipocalin 2 should be taken into consideration for optimizing clinical management strategies for inflammatory bowel disease with the use of EGCG treatment. (Am J Pathol 2016, 186: 912–926; http://dx.doi.org/10.1016/j.ajpath.2015.12.004)
against weight loss, reduce histologic damage, and increase survival in the dextran sodium sulfate (DSS)-induced acute murine colitis model.\textsuperscript{13} Likewise, long-term oral administration of EGCG was shown to reduce proinflammatory cytokines such as TNF, interferon \(\gamma\), and IL-6 in the colonic mucosa of DSS-induced colitic mice.\textsuperscript{3} However, the molecular mechanisms by which EGCG protects against gut inflammation are not completely elucidated.

Neutrophils, the most abundant circulating white blood cells, are the first responder cell type during inflammation, infection, and injury. These cells infiltrate the intestinal mucosa to reach the site of insult, subsequently undergo respiratory burst, and release myeloperoxidase (MPO;\textsuperscript{14} E.C.1.11.2.2). MPO is a heme-containing enzyme that uses hydrogen peroxide (\(H_2O_2\)) and halide ions to generate hypohalous acid, a potent oxidizing and antimicrobial agent.\textsuperscript{14} MPO is located within the neutrophils with the siderophore-binding innate immune protein called lipoclin 2 (|Lcn2; alias neutrophil gelatinase-associated lipoclin (NGAL)]. As part of the host response to inflammation, Lcn2 is released by both immune cells and intestinal epithelia,\textsuperscript{15} serving both as an epithelial growth factor\textsuperscript{16} and a chelator of bacterial siderophores, specifically enterobactin (Ent) with a high affinity (0.4 nmol/L).\textsuperscript{17} Thus, Lcn2 can deprive bacteria of iron and prevent their proliferation. Recently, we demonstrated that Ent is a potent inhibitor of MPO but loses its inhibitory properties when bound to Lcn2.\textsuperscript{18} Similar to Ent, EGCG was shown to reduce MPO activity\textsuperscript{19} and to form complexes with iron and Lcn2 in vitro.\textsuperscript{20} However, a detailed mechanism of EGCG-mediated MPO inhibition, the extent to which iron- and host-derived Lcn2 influence EGCG bioactivity, and the \textit{in vivo} functionality of these \textit{in vitro} observations remain unknown.

Iron is an essential trace metal that is required by both the host and the gut microbiota for their biological functions. Most circulating iron in the host are bound to proteins, although a small fraction remains unbound and is regarded as the labile or catalytic iron.\textsuperscript{21} These unbound iron molecules are highly reactive and readily participate in Fenton reaction in which free ferrous iron (Fe\(^{2+}\)) react with \(H_2O_2\) to produce ferric iron (Fe\(^{3+}\)).\textsuperscript{22} Fe\(^{3+}\) then reacts with \(H_2O_2\) in the Haber-Weiss reaction to generate hydroxyl radicals and Fe\(^{2+}\) that again participate in the redox cycle.\textsuperscript{23} The resulting cyclical overproduction of free radicals leads to increasing oxidative stress and damage, which contribute to disease such as IBD. Indeed, iron supplementation was shown to aggravate inflammation in murine models of colitis.\textsuperscript{24,25} Although EGCG was previously shown to be capable of binding and reducing Fe\(^{3+}\) to Fe\(^{2+}\),\textsuperscript{26} the effect of iron on the efficacy of EGCG in treating IBD remains unexplored.

In this study, we report the potent inhibitory property of EGCG on two similar heme-containing peroxidases, MPO and lactoperoxidase (LPO; EC: 1.11.1.7)\textsuperscript{26} \textit{in vitro} and provide evidence to demonstrate that EGCG also inhibits MPO activity \textit{in vivo} in the inflamed gut. The inhibitory properties of EGCG are abrogated in the presence of iron and/or when EGCG complexes with murine Lcn2 or its human ortholog NGAL. Spectral analysis with the use of LPO-\(H_2O_2\) as a model system revealed that EGCG prevented the peroxidase-catalyzed reaction, suggesting that its antioxidative properties may be the underlying mechanism of EGCG-mediated MPO/LPO inhibition. Collectively, our study documents a novel \textit{in vivo} mechanism for the protective bioactivity of EGCG during intestinal inflammation as being, at least in part, because of the inhibition of MPO, a process that is counter-regulated by iron and Lcn2.

**Materials and Methods**

**Reagents**

Human MPO, human NGAL, and murine Lcn2 were procured from R&D Systems (Minneapolis, MN). EGCG was obtained from two sources: Sigma-Aldrich (St. Louis, MO) and Taiyo Green Power (Jiangsu, China). Ferric chloride, \(H_2O_2\), and 4-aminobenzoic acid hydrazide (ABAH; a known inhibitor of MPO\textsuperscript{27,28}) were procured from Sigma-Aldrich. Bovine milk LPO was purchased from Worthington Biochemical Corp (Lakewood, NJ). Guaiacol (2-methoxyphenol) and ascorbic acid were obtained from Alfa Aesar (Ward Hill, MA). Ferric chloride, \(H_2O_2\), and 4-aminobenzoic acid hydrazide (ABAH; a known inhibitor of MPO\textsuperscript{27,28}) were procured from Sigma-Aldrich. Bovine milk LPO was purchased from Worthington Biochemical Corp (Lakewood, NJ). Guaiacol (2-methoxyphenol) and ascorbic acid were obtained from Alfa Aesar (Ward Hill, MA). Reagent grade DSS (mol. wt. = 36,000–50,000; lot no. M8667) was obtained from MP Biomedicals, LLC (Solon, OH). Bleomycin sulfate was procured from Biotang (Lexington, MA). K12 Escherichia coli strain was acquired from The E. coli Genetic Stock Center, Yale University (New Haven, CT).

**Mice**

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house in the animal facility at The Pennsylvania State University. All animal experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University.

**MPO and LPO \textit{in Vitro} Assay**

Human MPO and bovine milk LPO were reconstituted with 0.1 mol/L potassium phosphate buffer (pH 6.0) and stored at \(-80^\circ\text{C}\). MPO (12.5 \(\mu\text{g/mL}\), final concentration) or LPO (25 \(\mu\text{g/mL}\), final concentration) were preincubated with various concentrations of EGCG with or without iron or Lcn2/NGAL for 5 minutes at room temperature in 20-\(\mu\text{L}\) reaction volume. The reaction was initiated on the addition of 30 \(\mu\text{L}\) of 2.2 mmol/L \(H_2O_2\) and 50 \(\mu\text{L}\) of 100 mmol/L guaiacol. This assay is based on the principle that peroxidase oxidizes guaiacol in the presence of \(H_2O_2\) to a chromophore \(3,3'-\text{dimethoxy-4,4'-biphenyldiquinone}\). The change in absorbance at 470 nm was measured at 1-minute intervals over a period of 10 minutes. The reactions were performed in 96-well plates (Corning, Corning, NY) in
triplicate with appropriate vehicles throughout the study at pH 6.0 and 25°C. One unit of MPO or LPO activity is defined as the amount that increases absorbance at 470 nm by one per minute at 25°C, calculated from the initial rate of reaction with the use of guaiacol as the substrate. The final concentrations of MPO and LPO used were determined to be equivalent to 0.20 to 0.25 unit of activity.

**In Vitro Bacterial Killing Assay**

**In vitro** bacterial killing assay was performed as described by Atosuo and Lilius.30 Briefly, the MPO reaction mixture was prepared by first adding 5 μg/mL MPO with or without EGCG at various concentrations in sterile phosphate-buffered saline (PBS). The concentration of chloride ion in PBS is estimated to be approximately 0.138 mol/L, which serves as the substrate required by MPO for the generation of hypohalous acid. Hydrogen peroxide (30 μmol/L) was then added to the reaction mixture and incubated at room temperature for 5 minutes. Finally, *E. coli* (K12 strain) grown overnight in Lysogeny broth media at 37°C with shaking at 250 rpm were added to the reaction mixture (2.0 × 10⁶ colony-forming unit per mL). After 30 minutes of incubation at room temperature, the reaction mixture was serially diluted and plated on nonselective Lysogeny broth agar plates in triplicates. Plates were incubated overnight at 37°C, and bacterial colony-forming units on plates were quantified. All indicated concentrations above represent final system concentrations.

**Spectral Analysis**

LPO (1 mg/mL) was reconstituted in 500 μL of 0.1 mol/L phosphate buffer (pH 6.0) and incubated with either vehicle, 50 μmol/L EGCG, or 50 μmol/L ABAH for 5 minutes. The reaction was initiated at room temperature on the addition of 30 μmol/L H₂O₂. Spectra at 250 to 500 nm were recorded every 10 seconds with the use of CARY50BIO UV-Visible Spectrophotometer. Each spectrum represents an average of six scans taken in 1 second. All indicated concentrations above represent final system concentrations.

**CAS Assay**

Chrome Azurol S (CAS) agar plates were prepared according to protocol outlined by Schwyn and Neillands.31 CAS remains blue in color when complexed with iron but turns orange when iron is chelated by iron chelators/siderophores. Briefly, 1 μL of EGCG mixed with or without ferric chloride at indicated concentrations were placed on CAS plate and monitored for orange halo formation. Afreric Ent (Sigma-Aldrich) was used as a positive control.

**Fe²⁺-Dependent Bleomycin-Induced DNA Damage Assay**

The bleomycin assay was performed as outlined by Burkitt et al32 with the following modifications. EGCG (1 mmol/L) or ascorbic acid (1 mmol/L) was added to a mixture that contained calf 50 μg/mL thymus DNA (Sigma-Aldrich), 50 μL/mL bleomycin sulfate, 0.1 mol/L Tris/HCl (Sigma-Aldrich), 5 mmol/L MgCl₂·6H₂O (Sigma-Aldrich) in water treated with Chelex 100 (Sigma-Aldrich). The reaction was initiated by adding the mixture to a series of Fe³⁺ concentrations prepared in 96-well solid black plate (Corning). For control, the mixture was prepared as above but without Fe³⁺. After incubation at 37°C for 1 hour, the reaction was stopped by adding 0.1 mmol/L EDTA (Sigma-Aldrich). The fluorescent DNA-intercalating agent ethidium bromide (0.1 mmol/L; Sigma-Aldrich) was added to the mixture. Fluorescence readings were then taken (excitation 510 nm; emission 590 nm). In principle, the conversion of Fe³⁺ to Fe²⁺ is detected as bleomycin-induced DNA damage that is proportional to the amount of available Fe³⁺. Damage to DNA corresponds to the loss in the ethidium bromide—enhanced fluorescent signal. Therefore, the percentage of DNA damage was calculated by comparing the ethidium bromide—enhanced fluorescence readings from the sample group with the control group. All indicated concentrations above represent final system concentrations.

**DSS-Induced Colitis in Mice**

In experiment 1, 8-week-old male C57BL/6J mice (n = 4) were administered 1.6% DSS in drinking water over a period of 7 days. The induction of colonic inflammation was confirmed via fecal occult blood, diarrhea, and loss in body weight. On day 6, mice were treated with 25 or 50 mg EGCG/kg body weight by oral gavage. On day 7, mice were given similar doses of EGCG and euthanized 3 hours later via carbon dioxide asphyxiation.

In experiment 2, 8-week-old male C57BL/6J mice (n = 4) were treated with 1.6% DSS (in drinking water), and 5 mg EGCG/kg body weight by oral gavage once daily for 7 days. Mice were euthanized 3 hours after the last dose of EGCG.

In experiment 3, 8-week-old male C57BL/6J mice (n = 4) were administered DSS as described above and then treated with either 50 mg EGCG/kg body weight alone or EGCG + iron [EGCG (50 mg/kg body weight) coadministered with iron in a molar ratio of 1:6] on day 6 and day 7 (24 and 3 hours before euthanasia, respectively).

In all experiments, mice in the control groups received only regular drinking water (without DSS) and given PBS (vehicle) oral gavage. Blood samples were collected at the time of euthanasia in a BD microtainer (Becton Dickinson, Franklin Lakes, NJ), via retro-orbital plexus. Hemolysis-free serum samples were obtained after centrifugation and stored at −80°C until further use. Colonies were flushed gently with PBS to remove fecal matter, weighed, and collected for MPO assay. Briefly, colonies were homogenized in 0.5% cetyltrimethyl ammonium bromide in potassium buffer (pH 6.0), freeze-thawed three times, sonicated, and centrifuged.33 Clear supernatant fluids were collected and used for MPO assay as described above.
Cytokine Assays

Fecal Lcn2, serum Lcn2, and serum keratinocyte-derived chemokine CXCL1 (KC) were measured by duoset enzyme-linked immunosorbent assay kits from R&D Systems according to the manufacturer’s protocol.34

Quantitative Reverse Transcription-PCR

Mouse distal colons were collected in RNAlater (Sigma-Aldrich) and stored at –80°C. RNA was extracted from colon tissue with the use of Trizol reagent (Sigma-Aldrich) as described in the manufacturer’s protocol. DSS is known to interfere with PCR reactions; therefore, we purified RNA with the use of the lithium chloride procedure as described in Viennois et al.35 Purified RNA (0.5 μg) was used to synthesize cDNA for quantitative real-time RT-PCR (RT-qPCR) with the use of SYBR green (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer’s protocol. The following primers were used to assess gene expression: KC34 forward, 5′-TTGTGCCAAAAGAAGTGCG-3′, and reverse, 5′-TCACACGGCCTCCCCAC-3′; Lcn234 forward, 5′-AACGGGCTTTACGATGACTACG-3′, and reverse, 5′-CTTGCACATT-GTAGCTGTGACC-3′; TNF34 forward, 5′-ACTCCAGGGGTTGCTATGT-3′, and reverse, 5′-AG-TGTAGGCTGGCCAT-3′; nitric oxide synthase 236 forward, 5′-TTTGCTTCCATGCTAATGC-GAAAG-3′, and reverse, 5′-GCTCTGTTGAGGTCTAAAGGCTCCG-3′; 36B434 forward 5′-TCCAGGCTTTGGGCATCA-3′, and reverse, 5′-CTTTATTCAGCTGCACATCACTCAGA-3′. 36B4 was used to normalize relative mRNA expression with the use of 2−DDCT method. Fold change was determined by comparison with the untreated control group.

Histology and Immunohistochemistry

After euthanasia, Swiss rolls of colons were fixed overnight in 10% buffered formalin solution and stored in 70% ethanol. Colons were processed for paraffin embedding, and serial paraffin sections (5 μm) were collected and stained with hematoxylin and eosin. For neutrophil staining, the paraffin-embedded colon sections were stained with rat anti-mouse antibody specific to neutrophil marker Ly6G (NIMP-R14; Abcam, Cambridge, UK) with the use of a diaminobenzidine peroxidase substrate kit from Vector Laboratories (Burlingame, CA) according to the manufacturer’s instruction. An appropriate negative isotype control (rat IgG2b; Abcam) was included to omit nonspecific staining. To estimate neutrophil infiltration into the colonic mucosa, at least four images (size: 2592p × 1944p) of NIMP-R14−stained tissue were obtained.
The images were analyzed with the use of ImageJ software version 1.49 (NIH, Bethesda, MD; http://imagej.nih.gov/ij), and a scale of 5 pixels/μm was set on the basis of the image legend. The mucosa layer was defined as the area between the muscularis mucosae and the luminal surface of the colon. Diaminobenzidine–NIMP-R14 positive-stained cells were counted and expressed as neutrophils/mm². Histologic scoring on hematoxylin and eosin-stained colon sections was performed as in Cooper et al. Briefly, colonic inflammation was graded for intestinal lesions, inflammation, mucosa, and percentage of affected area in the scale of 0 to 4, corresponding to the degree of increasing severity.

Statistical Analysis

All in vitro experimental results were reproduced in at least three independent experiments performed in triplicates. The in vivo mice experiments were individual studies with sample size of 4 mice. All values in the results are expressed as means ± SEM. The significance of difference between different groups was determined by one-way analysis of variance, followed by post hoc Sidak/Dunnet multiple comparison test in case of three or more groups and unpaired Student t-test in case of two groups. P < 0.05 was considered as significant. GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA) was used to calculate statistical significance.

Results

EGCG Inhibits Heme Peroxidases in a Dose-Dependent Fashion

EGCG was previously demonstrated to exhibit antioxidant and anti-inflammatory properties, although the exact mechanisms underlying such properties are not clearly understood. In this study, we tested whether EGCG could affect the bioactivity of neutrophil MPO, a pro-oxidant enzyme associated with acute inflammation. ABAH, which is a known MPO inhibitor, was used as the positive control. EGCG inhibited MPO activity in vitro in a dose-dependent manner (Figure 1A). EGCG also dose dependently inhibited the activity of LPO (Figure 1B), a heme-containing peroxidase with properties similar to MPO. In addition, EGCG mediated its inhibition on

Figure 2  Ferric iron-bound EGCG loses its ability to inhibit MPO and LPO activity. A: EGCG was preincubated with or without Fe³⁺ at indicated molar ratio and then 1 μL of the mixture was placed on CAS plate. Formation of orange halo indicates that EGCG chelated the iron from the CAS plate. Ent and FeCl₃ were used as positive and negative control, respectively. In the bleomycin-detectable Fe³⁺ assay, EGCG or ascorbic acid (positive control) was incubated with indicated doses of Fe³⁺ in the reaction mixture for 1 hour at 37 °C. B: Line graphs represent the conversion of Fe³⁺ to Fe²⁺, which was quantitated as Fe²⁺/Fe³⁺-dependent bleomycin-induced DNA damage. C and D: Bar graphs represent inhibition of MPO (C) and LPO (D) activity by 50 μmol/L EGCG that was preincubated with Fe³⁺ for 5 minutes at the indicated molar ratio before the assay was initiated. Results are expressed as means ± SEM. Assays were performed in a 96-well plate in triplicates and are representative of three independent experiments. * P < 0.05. CAS, chrome Azurol S; EGCG, (−)-epigallocatechin-3-gallate; Ent, enterobactin; Fe²⁺, free ferrous iron; Fe³⁺, ferric iron; FeCl₃ iron(III) chloride; LPO, lactoperoxidase; MPO, myeloperoxidase.
MPO activity even after the MPO reaction was initiated (Figure 1C). The ability of EGCG to inhibit MPO and LPO activity was observed over a range of physiologically relevant pH (5.0 to 7.4; data not shown) and temperatures (25 to 40°C; data not shown).

The end product of MPO-catalyzed reaction, hypohalous acid, acts as a potent antibacterial agent by halogenating bacterial cell membrane components. To confirm that EGCG-mediated inhibition could reduce the generation of hypohalous acid by MPO, we measured the viability of K12 E. coli exposed to MPO + H2O2 in the presence or absence of EGCG. Our study found that EGCG dose dependently reduced MPO + H2O2-mediated bacterial killing compared with the control (Figure 1D), suggesting that EGCG could prevent cellular damage induced by MPO and its end products.

**Fe³⁺-Bound EGCG Fails to Inhibit Heme Peroxidase Activity**

Because EGCG is known to chelate iron,38 we next asked whether the presence of iron could affect the ability of EGCG to inhibit MPO and LPO. First, we tested whether the EGCG used in this study is iron free and could bind to iron. EGCG chelated iron from CAS plate which resulted in a distinct orange halo formation (Figure 2A). Ent was used as a positive control. We conducted this experiment with EGCG procured from two different sources, and the results were consistent (data not shown), confirming that the EGCG used in this study is iron free. However, this halo-forming effect was lost in the presence of increasing concentrations of ferric iron (Figure 2A). When EGCG was mixed with high concentration of ferric iron, we observed the presence of a black-colored precipitate in the solution and also on the CAS plate, suggesting that EGCG is capable of reacting with iron and likely converts Fe³⁺ to Fe²⁺ iron.39 To ascertain this possibility, we performed a modified bleomycin-detectable iron assay to evaluate the redox reactions of EGCG with iron. Ascorbic acid, as a reducing agent and a positive control, reduces ferric iron and induces oxidative DNA damage even at a low iron concentration. Compared with ascorbic acid, EGCG catalyzed the conversion of Fe³⁺ to Fe²⁺ only in the presence of higher Fe³⁺ concentration, beginning with the molar ratio EGCG:Fe³⁺ of 1:1 (Figure 2B). More importantly, the presence of EGCG:Fe³⁺ in the molar ratio of 1:5 and 1:10 significantly mitigated the ability of EGCG to inhibit MPO and LPO activity (Figure 2, C and D), which highlight ferric iron as a potential counter-regulator of EGCG function.
Innate Immune Protein Lcn2 Prevents EGCG from Inhibiting Heme Peroxidases Activity

One of the well-established functions of Lcn2 is to chelate the bacterial siderophore Ent, which is rich in phenolic groups. Because both MPO and Lcn2 are present in the neutrophils and are robustly expressed during intestinal inflammation, we next asked whether Lcn2/NGAL can influence EGCG-mediated inhibition of MPO and LPO, especially because EGCG is also rich in phenolic groups. Human NGAL prevented EGCG from inhibiting MPO and LPO activity in a dose-dependent manner (Figure 3, A and B). Similar results were observed when the experiments were performed with Lcn2, which is the murine ortholog of the human NGAL (Figure 3, C and D).

EGCG Inactivates Heme Peroxidase-Catalyzed Reaction

To elucidate the underlying mechanism of EGCG-mediated MPO/LPO inhibition, we performed spectral analysis of LPO-catalyzed reaction with H2O2 in the presence or absence of EGCG. Because of technical limitation and unavailability of MPO supplied at the amount required for experiments with spectral analysis, we therefore used LPO as our model system. Spectral analysis of LPO alone showed a λ_max of 412 nm (Figure 4A), which is characteristic for the heme moiety in the enzyme. The addition of EGCG or ABAH (positive control; known inhibitor of LPO and MPO) to LPO did not induce any change in the λ_max, suggesting that EGCG and ABAH do not directly associate with the heme moiety of the peroxidase. After spectral analysis, we confirmed that the LPO incubated with EGCG is completely inactivated via LPO assay described previously (data not shown).
EGCG Inhibits MPO Activity in the Inflamed Gut of Colitic Mice

We tested whether the EGCG-mediated inhibition of MPO in vitro could be replicated in the DSS-induced murine model of colitis. Treatment with EGCG (25 or 50 mg/kg body weight) dose dependently reduced colonic MPO activity compared with DSS-only control (Figure 5A). However, it is possible that the observed decrease in colonic MPO activity could be due to decreased neutrophil infiltration, secondary to other anti-inflammatory effects of EGCG. Hence, we examined the differences in neutrophil infiltration by immunohistochemistry. Although DSS-only treatment increased neutrophil infiltration compared with control, EGCG treatments did not significantly mitigate those effects (Figure 5, B and D) compared with the DSS-only treatment, demonstrating that the observed reduced colonic MPO activity was attributable to inhibition by EGCG and not due to changes in neutrophil infiltration. In this experiment, however, oral administration of only two doses of EGCG (24 and 3 hours before euthanasia) did not result in observable improvement in the inflamed colon histology (Figure 5C).

EGCG Attenuates DSS-Induced Colitis in Mice

Iron chelator deferoxamine was previously demonstrated to induce increased expression and secretion of proinflammatory chemokine IL-8 by intestinal epithelial cells.\textsuperscript{31}

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**Figure 5** EGCG inhibits colonic MPO activity in the inflamed gut of colitic mice. DSS-induced colitic mice were orally gavaged with 25 or 50 mg EGCG/kg bodyweight at 24 and 3 hours before euthanasia. Colons were collected and processed for MPO assay. A–C: Bar graph represents the colonic MPO activity (A), neutrophil count (B), and colon histologic score (C). D: Images represent immunohistochemistry staining for neutrophil marker Ly6G. Results are expressed as means ± SEM. n = 4 DSS-induced colitic mice. *P < 0.05. Original magnification: ×100 (D, upper row); ×400 (D, lower row, enlarged from corresponding boxed areas above.). DSS, dextran sodium sulfate; EGCG, (−)-epigallocatechin-3-gallate; MPO, myeloperoxidase.
Hence, we tested whether EGCG, also an iron chelator, would affect the basal expression of inflammatory markers in the gut of healthy mice. We orally administered 50 mg EGCG/kg body weight to 8-week-old male C57BL/6 mice at 24 and 3 hours before euthanasia and then cultured colon sections ex vivo in incomplete Dulbecco’s modified Eagle’s medium for 24 hours. However, we observed no difference in the levels of secreted Lcn2 and KC (murine homolog of human IL-8) in the culture media when comparing the untreated and EGCG-treated group (data not shown), indicating that EGCG treatment does not affect basal expression of inflammatory markers in healthy mice.

Having established that EGCG could inhibit MPO activity in vivo, we next asked whether EGCG can attenuate intestinal inflammation as well. Although treatment with daily doses of 5 mg EGCG/kg body weight for 7 days did not prevent weight loss in colitic mice (Figure 6A), the treatment prevented splenomegaly and colon shortening that are associated with DSS-induced colitis (Figure 6, B–D). These mice displayed a less severe colitis as reflected by the significantly reduced levels of colonic MPO activity, serum KC, serum Lcn2, and fecal Lcn2 (Figure 6, E–H), all of which are sensitive markers of intestinal inflammation. Further, the colonic expression for proinflammatory genes that encode KC, Lcn2, nitric oxide synthase 2, and TNF were significantly reduced in EGCG-treated colitic mice (Figure 6, I–L). However, we did not observe significant histologic changes between colon of DSS-only and EGCG-treated group (Figure 6, M and N).

Oral Administration of Iron-Saturated EGCG Fails to Inhibit MPO in Vivo in Acute Colitis

Our in vitro results demonstrated that iron-saturated EGCG failed to inhibit MPO- and LPO-catalyzed reactions. We next examined whether this effect could be
recapitulated in vivo by treating DSS-induced colitic mice with either vehicle, 50 mg/kg body weight EGCG alone, or EGCG + iron (50 mg/kg bodyweight with iron co-administered at EGCG:iron molar ratio of 1:6). Control mice received only regular drinking water. A–I: The following variables were analyzed: body weight (A), gross colon (B), spleen weight (C), colon length (D), MPO activity at the proximal colon (E) and distal colon (F), serum KC (G), serum Lcn2 (H), and fecal Lcn2 (I). Quantitative real-time RT-PCR analysis was used to quantify mRNA expression of KC (J), Lcn2 (K), and TNF (L). mRNA values are represented as fold-change normalized to 36B4 housekeeping gene. M and N: Hematoxylin and eosin staining of colon Swiss rolls (M) and their corresponding histologic score (N). Results expressed as means ± SEM. n = 4 DSS-induced colitic mice. *P < 0.05. Original magnification: ×100 (M, upper row); ×400 (M, lower row). DSS, dextran sodium sulfate; EGCG, (+)-epigallocatechin-3-gallate; KC, keratinocyte-derived chemokine CXCL1; Lcn2, lipocalin 2; MPO, myeloperoxidase; TNF, tumor necrosis factor.
chelating iron from the heme moiety of MPO and LPO, but this was not the case. Contrary to our initial hypothesis, EGCG mediates its antioxidant effect without directly interfering with the heme moiety of the peroxidase. Spectral analysis on EGCG-mediated inhibition of LPO, a heme-containing enzyme that has similar function to MPO, reveals that EGCG reverts the peroxidase radical intermediate o xoiron back to its native inactive state, possibly by donating electrons to oxoiron. O xoiron is required to react with halide ion to generate hypohalous acid. The reactivity of hypohalous acid to heme iron (including those in MPO) was shown to induce heme degradation and the release of free or reactive iron leading to a cascade of free radical generation. In lieu of this, the inactivation of oxoiron by EGCG would effectively negate the production of hypohalous acid by MPO and LPO. By limiting MPO activity, EGCG could play a role in preventing collateral tissue damage by hypohalous acid during acute intestinal inflammation.

The beneficial effects of EGCG in treating gut inflammation were demonstrated in mouse models of colitis. In one study, EGCG-enriched diet was shown to induce down-regulation of inflammation-associated genes such as IL1B, TNFA, interferon γ gene, Toll-like receptor 2 gene, and CD14 in spontaneously colitic Mdr1a–/– mice. It is thought that EGCG mediates its therapeutic effects by modulating gene expression via inhibition of various transcription factors, including NF-kB, Sp1, activating protein-1, STAT1, STAT3, and forkhead box O1. Aside from this, EGCG not only inhibits NADPH oxidase, which produces reactive oxygen species (ROS), but also scavenges ROS and reduces oxidative stress. ROS is one of the main factors that contribute to IBD pathology, and its elimination by EGCG may alleviate gut inflammation. Hence, the ability of EGCG to inhibit MPO and other neutrophils’ pro-oxidant enzymes could provide an additional layer of protection, which perhaps could be one of the primary effects of EGCG that leads to the secondary down-regulation of downstream proinflammatory responses.

Interestingly, EGCG is also demonstrated to inhibit neutrophil elastase and to impair the migration of neutrophils in response to the chemoattractant IL-8. However, we did not observe a difference in the amount of neutrophils in the mucosa between the untreated and EGCG-treated colitic mice, despite the apparent reduction of colonic MPO activity in the latter. It is likely that the neutrophils were already present in mucosa before EGCG treatment or that the dosage of EGCG that we used in this study was insufficient to induce observable reduction in neutrophil infiltration. In addition, the low EGCG doses used in this study may also explain the lesser degree of protection against colitis compared with previous reports that used higher doses of EGCG. Regardless, the effectiveness of EGCG in treating IBD was shown to be comparable with the widely used anti-colitogenic drug sulfasalazine in the murine model of DSS-induced colitis. Indeed, the

**Figure 8** Potential mechanism by which EGCG mediates antioxidant effects that could alleviate gut inflammation. During inflammation, neutrophils release a plethora of pro-oxidant enzymes; one such enzyme is MPO whose bioactivity is associated with flares of inflammatory bowel disease. EGCG potently inhibits the activity of MPO and therefore reduces the level of oxidative stress in the inflamed gut. However, the beneficial MPO inhibition by EGCG is counter-regulated by iron and host Lcn2/NGAL. Altogether, the present study unravels the complex regulation of EGCG on MPO activity and its counter-regulation by iron and host Lcn2/NGAL. EGCG, (−)-epigallocatechin-3-gallate; Fe³⁺, ferric iron; Lcn2, lipocalin 2; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin.
The potential use of EGCG to treat gut inflammation and other diseases has garnered a great deal of attention, leading to its current inclusion in human clinical studies. The efficacy of EGCG in human clinical trials remains somewhat inconclusive because EGCG treatments were found to aggravate gut inflammation rather than to alleviate the disease in several cases. Although it is not completely understood, many studies implicate that this could be due to the pro-oxidant properties of EGCG in the presence of Fe$^{3+}$. EGCG was previously shown to form complexes with Fe$^{3+}$ and to participate in the Fenton reaction. The reduction of Fe$^{3+}$ to Fe$^{2+}$ accelerates the formation free hydroxyl radicals and ROS that are detrimental to the gut. Interestingly, high doses of EGCG were shown to induce Fenton reaction and to generate more ROS, whereas low doses of EGCG scavenges ROS in vitro. Accordingly, several animal studies also demonstrated that high doses of dietary EGCG tend to induce a pro-oxidative response that aggravates gut inflammation in vivo. Although we did not observe the purported detrimental effects of EGCG in this study, our in vitro findings do demonstrate that iron at 1:1 molar ratio induces EGCG to switch from being an antioxidant into one that is pro-oxidant. In addition, the presence of excess/dysregulated iron can counter the beneficial effect of EGCG by additionally abrogating its ability to inhibit MPO and LPO activity. This suggests that the presence of iron-rich conditions could potentially alter EGCG bioactivity and may shift it from being an inhibitor of MPO into a harmful pro-oxidant. Hence, the consumption of diets rich in iron or oral iron supplements to treat iron deficiency-induced anemia in IBD at the same time as EGCG administration may be detrimental. Iron is an essential nutrient to most bacteria, excluding Lactobacillus and Borrelia species. The hypothermic conditions induced by acute inflammation in the gut may allow growth of bacteria that could better adapt to iron-poor conditions. The ability of EGCG to bind Fe$^{3+}$ and its reduction to Fe$^{2+}$ may have substantial influence over bacterial iron acquisition because bacterial siderophores have high affinity specific to Fe$^{3+}$ but not Fe$^{2+}$. In addition, EGCG can prevent Enterococcus faecalis biofilm formation via EGCG-Fe$^{2+}$ mediated hydroxyl radical formation. Further, the chelation of EGCG + Fe$^{3+}$ binary complex by host antibacterial innate immune protein NGAL/Lcn2 may add another layer of complexity to the acquisition of iron by resident bacteria in the inflamed gut. However, the iron-binding and oxidative properties of EGCG may be beneficial in exerting antibacterial pressure in the inflamed gut and perhaps play a role in alleviating disease by modulating gut bacteria composition and growth. Yet, this may also result in a rapid decrease in beneficial bacteria and overgrowth of opportunistic pathogens (eg, E. coli), which can express Lcn2/NGAL-resistant stealth siderophores. Further studies are warranted, given our current limited knowledge on the interplay between EGCG and gut microbiota in the inflamed gut.

A study by Barasch and colleagues demonstrated that NGAL could form a stable complex with iron-bound EGCG and could suppress its pro-oxidant properties in vitro, highlighting the first molecule of plant origin to form complexes with NGAL. Although the presence of NGAL may be beneficial in preventing the adverse pro-oxidative effect of EGCG, the EGCG sequestration by NGAL could also eliminate its therapeutic properties. As confirmed in our present study, both human NGAL and murine Lcn2 counter-regulate EGCG-mediated inhibition of MPO and LPO activity in a dose-dependent fashion. It would appear that NGAL inhibits EGCG optimally at a molar ratio NGAL:EGCG of 1:1, which is consistent with a previous report that one molecule of NGAL can bind to one molecule of EGCG. NGAL and its murine ortholog Lcn2 are known to be significantly elevated in the inflamed gut of humans and mice, respectively. Therefore, NGAL/Lcn2 presents a possible counter-regulatory role that potentially mitigates the efficacy of EGCG in treating IBD. Future studies should consider the levels of circulating/mucosal NGAL/Lcn2 in designing an optimal dose of EGCG to be administered to patients with IBD.

Overall, our study demonstrates that inhibition of MPO by EGCG serves as a mechanism by which EGCG confers protection against gut inflammation. Moreover, as shown in Figure 8, the beneficial functions of EGCG potentially depend on the environment in the gut (ie, dietary iron and luminal NGAL/Lcn2, both of which counter-regulate EGCG bioactivity). Thus, our data provide the experimental basis to take these counter-regulatory factors into consideration to further develop EGCG as a potential therapeutic agent to treat human IBD.

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References


3. Oz HS, Chen T, de Villiers WJ: Green tea polyphenols and sulfasalazine have parallel anti-inflammatory properties in colitis models. Front Immunol 2013, 4:132


6. Angelo LS, Kurzrock R: Turmeric and green tea: a recipe for the modulation of angiogenesis and growth via inhibiting the activation of HIF-1alpha and NFkB, and VEGF expression. Vase Cell 2013, 5:9


11. Yeoh et al


36. Viennois E, Chen F, Laroui H, Baker MT, Merlin D: Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. BMC Res Notes 2013, 6:226


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50. Kimm KC, Park KI, Chung HT, Jun CD: Epigallocatechin-3-gallate mediates T cellular NF-kappa B inhibition and (−)-epigallocatechin gallate, and thea
e

Iron and Lcn2 Abrogate EGCG Bioactivity

73. Lee P, Tan KS: Effects of Epigallocatechin gallate against Entero-


